SEROLOGICAL AND MOLECULAR DETECTION OF DIFFERENTIAL INFECTIONS OF BUNCHY TOP AND MOSAIC CAUSING VIRUSES IN TISSUE CULTURE PLANTLETS OF ABACA (*Musa textilis* Née)

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ABSTRACT

The abaca planting materials are usually propagated through tissue culture, and its production requires reliable virus indexing protocol to ensure that plants are indeed virus-free. This study aimed to establish a reliable protocol for virus indexing of abaca tissue cultures using serological and molecular methods considering differential infections during the tissue culture process. The objectives were to determine the differential infection of plantlets in tissue culture line and plantlets obtained following subsequent *in vitro* and *ex vitro* cultures. The materials were plantlets of *in vitro* and *ex vitro* culture lines obtained from infected tissue explant. In this study, differential infection of *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV) and *Sugarcane mosaic virus* (SCMV) were detected by enzyme-linked immunosorbent assay (ELISA) among plantlets obtained from a single tissue culture line, and in plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. The differential infection resulted to inconsistency of virus detection by ELISA. Infection of *in vitro* abaca cultures occurred mostly as mixed infection of two or three viruses. The differential infection of BBTV was confirmed by PCR detection. The sensitivity of BBTV detection by PCR was affected by the dilution of template DNA.

Key Words: bract mosaic, sugarcane mosaic, polymerase chain reaction, enzyme-linked immunosorbent assay, virus indexing

INTRODUCTION

Abaca (*Musa textilis* Nee) also known worldwide as the Manila hemp is indigenous in the Philippines, and is one of the most economically important crops in the country. The Philippines dominates abaca fiber production and supplies about 85% of the total abaca fiber worldwide. In 2016, the country produced a total of 496,069 bales in 125.5 kg of abaca fibers, with Bicol Region as the top producer (PhilFIDA 2016). The production of abaca is seriously affected by virus diseases (Thomas et al. 2003) causing significant losses to the industry These diseases include bunchy top, bract mosaic and abaca mosaic which have taken their toll on many farmers and persistently devastated abaca plantations in abaca producing regions in the Philippines (FIDA 2011). Abaca bunchy top is known to be caused by *Banana bunchy top virus* (BBTV) which is transmitted by aphid, *Pentalonia*

nigronervosa, in a persistent, circulative, non-propagative manner (Magee 1953). In 2008, a distinct virus species, the *Abaca bunchy top virus* (ABTV) has also been found associated with the disease (Sharman et al. 2008). Bunchy top infected plants are stunted which produces undersized suckers with short, narrow, stiff and upcurled leaves, and chlorotic to necrotic leaf margins (Ocfemia et al. 1930; Raymundo 2000; Bajet and Magnaye 2002). Abaca mosaic is caused by the aphid transmitting abaca strain of *Sugarcane mosaic virus* (SCMV) with symptoms of chlorosis and mosaic on leaves (Eloja et al. 1962; Eloja and Tinsley 1963; Bajet and Magnaye 2002; Gambley et al. 2004). *Banana bract mosaic virus* (BBrMV) also infects abaca (Espino et al. 1990; Magnaye and Espino 1990; Sharman et al. 2000). The virus is transmitted in a non-persistent manner by *P. nigronervosa*, *Aphis gosypii* and *Rhopalosiphum maidis* (Magnaye and Espino 1990, Muñez 1992). The BBrMV causes discontinuous streaks on the bract, spindle-shaped streaks on the petiole, and mottling on the pseudo stem (Rodoni et al. 1997). All of these viruses can also be transmitted through vegetative propagation.

Since 1992, the Philippine government, through the Philippine Fiber Industry Development Authority (PhilFIDA), implemented the abaca rehabilitation program in its effort to effectively manage the virus diseases affecting abaca. The rehabilitation program is part of the production support services by PhilFIDA which include: eradication of infected plants and replanting, development of new abaca areas, facility upgrading, and abaca disease management project. In support of the rehabilitation program, mass propagation of planting materials is needed. The meristem/shoot tip culture technique has been developed for abaca to rapidly propagate disease-free planting materials and produce of cheaper plantlets with high survival rate in the field. This has been adopted by other research laboratories throughout the country, and has long been transferred to the farmer's fields (Aspuria 2003). However, it is crucial to ensure that the source of the explant and the derived plantlets are virus-free.

The BBTV, SCMV and BBrMV have been shown to be transmitted through tissue culture (Diekmann and Putter 1996, Drew et al. 1989 and 1992, Ramos and Zamora 1990, Wu and Su 1991). BBTV is readily transmitted through tissue culture in banana cultivars, and the virus is efficiently detected by ELISA (Thomas et al. 1995). BBrMV is also detectable in *in vitro* cultures (Hwang and Su 1998). For abaca tissue culture, plantlets are obtained using meristematic tissue from a healthy mother plant. The plantlets are separated, subsequently subcultured for further propagation, and then indexed to ensure that they are virus-free. However, a standard protocol for virus indexing of abaca tissue culture needs to be established in the country. Current virus indexing of tissue culture plantlets involves testing the source of the explant and the representative plantlets collected either at the *in vitro* or *ex vitro* stages. However, in case the virus has not been detected in the explant and then used for tissue culture, it is possible that the plantlets obtained from a single explant would be differentially infected. The differential infection is attributed to the irregular distribution and movement of the virus in actively growing meristematic tissues as shown for BBTV (Thomas et al. 1995). The differential infection of plantlets during tissue culture and the subsequent cultures may affect the reliability of virus indexing.

This study sought to establish a reliable protocol using serological and molecular methods for virus indexing of abaca tissue cultures considering the differential virus infection of tissue culture plantlets. The objectives were to determine the differential infection among plantlets of tissue culture lines and among plantlets obtained following subsequent *in vitro* and *ex vitro* cultures.

MATERIALS AND METHODS

The study was conducted at the Plant Virology Laboratory, IWEP (formerly Crop Protection Cluster) and the Plant Tissue Culture Laboratory, ICropS, formerly Crop Science Cluster (CSC), University of the Philippines Los Baños (UPLB) in 2013-2014.

Source of *in vitro* abaca cultures

The source of *in-vitro* abaca cultures cv. Inosa was provided by the Plant Tissue Culture Laboratory, CSC, UPLB. The plantlets were 2-3 year-old established stock cultures acquired from the PhilFIDA in Tacloban, Leyte, and the National Abaca Research Center (NARC) in Baybay City, Leyte. The established cultures were sub-cultured in modified Murashige and Skoog (MS) (1962), a standard multiplication medium supplemented with 5 ppm benzyladenine (BA). There were eight stock cultures from PhilFIDA namely, 1514, H61122, 1541, D21511, L51417, N21478, V1128 and E21516, and these were considered in this study as batch 1 samples. The 10 stock cultures from NARC were ST10, ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20 and ST22, and these were considered as batch 2 samples. Each stock culture was considered as a tissue culture line, e.g Line ST10, ST11, ST12 etc. Shoot tissue collected from batch 1 and 2 samples was subcultured for three consecutive cycles. After the third subculture, the plantlets were transferred to rooting media, and then maintained as *ex vitro* cultures in the screenhouse. Leaf samples from plantlets of each line (batch 1 and 2) were collected for virus assay at the first and second *in vitro* culture, and then at the *ex vitro* stage. The samples were tested for the presence of BBTV by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). Both methods have been established for detection of abaca viruses (Hwang and Su 1998, Thomas and Dietzgen 1991, Furuya et al. 2006, Mahadev et al. 2013). The presence of BBrMV and SCMV were tested by ELISA.

Sub-culture of plantlets

Multiplication of shoots. Plantlets taken from the abaca stock cultures were inoculated to freshly-prepared MS basal medium containing salts (macro and micronutrients) and Nitsch and Nitsch vitamins (1969) supplemented with plant growth regulators (1uM indole-3-acetic acid (IAA) and 10uM benzyadenine purine (BAP), 3 ppm benzyladenine, and 3% sucrose, adjusted to pH 5.8 and solidified using 0.55% plant tissue culture (PTC) agar (Pronadisa). Two to three shoots per plantlet were subcultured for tissue proliferation in a period of three months**.** The first *in vitro* subculture plantlets were generated after one month, and then these were used as the source to produce the second subcultured plantlets one month thereafter. The second subcultures were again used as the source to produce the third subculture plantlets one month thereafter. The subcultured plantlets consisted of the tissue culture lines, with each line corresponds to the stock cultures described above. The plantlets were observed for the presence of virus-like symptoms, such as mosaic and chlorosis.

***Root induction*.** After 3 months of subculture in the multiplication medium, the plantlets were transferred to rooting medium to promote the proliferation of roots. The rooting medium was composed of MS basal salts, Nitsch and Nitsch vitamins, 0.25 ppm indole-3-butyric acid (IBA), 6% sucrose, 0.25% activated carbon solidified with 0.55% PTC agar (Pronadisa) at pH adjusted to 5.8. The plantlets were cultured in the rooting medium for 1 month and then acclimatized for 2 weeks. Acclimatized plantlets were transferred to potting medium (1 sterilized garden soil: 3 coir dust), and these served as the *ex vitro* cultures. The plantlets were pre-treated with fungicide, and were maintained inside an insect-proof net cage to prevent virus transmission by the aphid vector.

Virus detection

The plantlets derived from the first and second *in vitro* subculture, and from *ex vitro* culture of each abaca line were assayed for the presence of abaca viruses. For each line, the number of samples subjected for virus indexing depended on the availability of plantlets having adequate leaf tissue for sampling. At the first *in vitro* culture stage, BBTV detection consisted of 23 total samples collected from plantlets of tissue culture lines in batch 2 samples, while BBrMV and SCMV detection consisted of 32 total samples collected from batch 1 and 48 of batch 2 samples.

Analysis of differential infection of BBTV in plantlets following subculture consisted of samples of the first *in vitro* and *ex vitro* cultures. BBTV detection at the *ex vitro* stage consisted of 20 total samples from ST10, ST11, ST12, ST13, ST17, ST18, ST19, ST20 and ST 22 lines. For ST10

line, the *ex vitro* culture samples were collected from plantlets, ST10(4-1), ST10(8-1) and ST10(10-1) which were derived from the subcultured plantlets ST10(4), ST10(8) and ST10(10), respectively. Due to limited samples available for testing, fewer plantlets from the *ex vitro* stage were tested. Analysis of BBrMV differential infection following subculture consisted of samples from the first and second *in vitro* cultures. Detection of BBrMV and SCMV consisted of 17 total samples from ST10, ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20 and ST 22 lines. For ST10 line, the second *in-vitro* culture sample was collected from plantlet ST10(8-1) derived from the subcultured plantlets ST10(8) of the first *in vitro* culture.

Direct ELISA. The Plate Trapped Antigen (PTA) ELISA was employed for BBrMV and SCMV detection using the commercial kit from Agdia, and following the manufacturer's protocol with some modifications. Instead of using the Agdia general extraction buffer, the tissue samples were homogenized at 1:5 dilution in Tris-Na-DIECA buffer (Bajet and Magnaye 2002). The buffer was added at the start of grinding to avoid tissue oxidation. The succeeding steps then followed the Agdia protocol. Samples were considered positive to the virus when the absorbance reading was above the threshold value, which was twice the average of the absorbance of three healthy control samples.

Indirect ELISA. Detection of BBTV was conducted by indirect PTA ELISA. Commercial BBTV polyclonal antibody (Agdia) was used following the protocol described by Su (1999) and Bajet and Magnaye (2002).

Polymerase chain reaction. Total DNA was extracted following the protocol by Su (1999) with some modifications. The presence of BBTV was also detected by polymerase chain reaction (PCR) while the presence of BrMV and SCMV were not tested in this study. Detection of BBTV by PCR followed the procedure described by Thompson and Dietzgen (1995) using BBTV specific primers, BBT-1 and BBT-2 designed to amplify the DNA-R component of the viral genome with an expected amplicon size of 349 bp. The negative samples were retested using 1:10 diluted DNA template. The reaction mixture was subjected for amplification in a Veriti® 96-well thermal cycler (Applied Biosystems) with the following conditions: initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 1 min, extension at 72° for 1 min, and a final extension at 72°C for 3 min (Thompsom and Dietzgen 1995). The PCR product was subjected to gel electrophoresis to check for the presence of amplified DNA. The DNA was stained using a dye (GelRed™) and then viewed in a gel documentation system (AlphaImager® Mini - Alpha Innotech).

RESULTS AND DISCUSSION

This study showed the differential infection of BBTV, BBrMV and SCMV among abaca tissue culture plantlets obtained from a single tissue culture line, and among plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. Although the plantlets of each line were derived from a single explant, these plantlets were found to be differentially infected. Likewise, the plantlets obtained from a single plantlet following subsequent culture were also differentially infected.

Virus-like symptoms in abaca tissue cultures

Virus-like symptoms such as deformation, vein chlorosis and swelling were observed on the leaves of some *in vitro* cultures (Figure 1a-b). Vein necrosis which is not a typical virus disease symptom was also observed (Figure 1c-d). However, these symptoms cannot be attributed at all to virus infection since these were observed even on the virus-free plantlets, while not all virus-infected plants had these symptoms. For instance, although some *in vitro* cultures were positive to BBTV, none of these symptoms were observed. The plantlets were also tested for BBrMV and SCMV infection and the presence of symptoms did not correspond to virus infection. The symptoms observed may be morphological and physiological disorders brought about by the process of tissue culture.

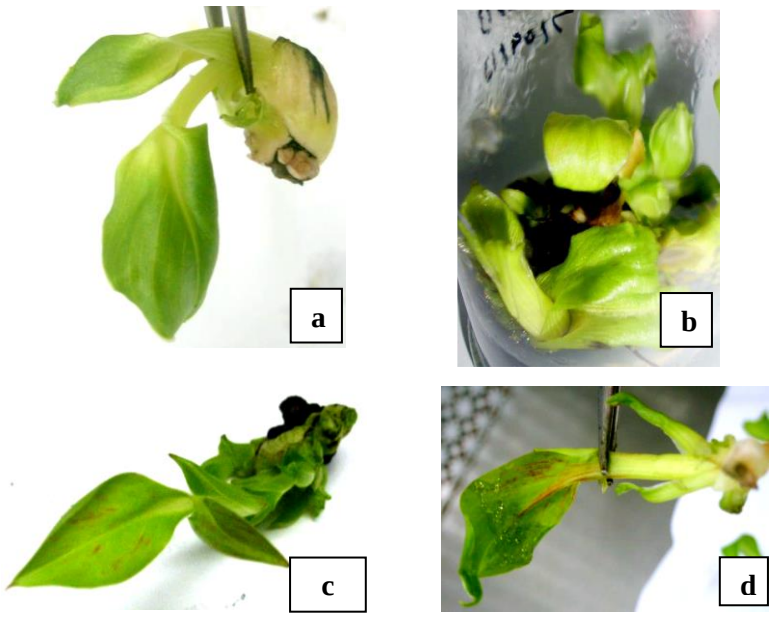

Fig. 1. Symptoms observed in *in-vitro* cultures of abaca. a) vein swelling; b) leaf deformation; and c-d) vein necrosis.

Viruses detected in abaca tissue culture lines by enzyme-linked immunosorbent assay

The BBTV, BBrMV and SCMV were detected by ELISA, but the presence of the virus was differentially detected among plantlets derived from a single tissue culture line (Table 1). The plantlets of tissue culture lines from batch 1 and batch 2 samples with each line derived from a single explant were differentially infected. The eight out of ten ST lines (batch 2 samples) were positive to BBTV, in which all plantlets of five lines ST10, ST11, ST12, ST17, and ST 20 were consistently infected while the plantlets of other lines ST14, ST18, ST19 and ST22 were differentially infected. For instance, one out of two plantlets of line ST14 was positive to BBTV while the other plantlet was negative. The virus was not detected in all plantlets of lines ST13 and ST19.

The BBrMV was consistently detected in all plantlets of the seven lines (1514, H61122, 1541, D21511, L51417, N21478 and E21516) but not V1128 line (Table 1). On the other hand, the plantlets of the ST lines were differentially infected with BBrMV in which all plantlets of five lines (ST10, ST11, ST13, ST19, ST20) were all infected while those of the other lines ST12, ST14, ST17, ST18 and ST22 were differentially infected. Plantlets were also differentially infected with SCMV (Table 1). All plantlets of four (H61122, L51417, N21478, V1128) out of eight lines were consistently positive to SCMV. Plantlets of two ST lines (ST10, ST22) were consistently infected with SCMV while those of the other lines (ST11, ST12, ST13, ST14, ST17, ST18, ST19, ST20) were differentially infected. The BBTV, BBrMV and SCMV were detected mostly as mixed infections.

Infection of *in vitro* abaca cultures occurred mostly as mixture of two or three viruses. Like in banana, mixed virus infections in abaca commonly occur in the field. Abaca plants from different abaca growing areas were found to be mixed infected with BBTV and BBrMV (Sta. Cruz et al. 2016), and with BBrMV and SCMV (Gambley et al. 2004, Sta. Cruz et al. 2016). Thus, it is important to ensure that the explants to be used for tissue culture propagation are tested for infection with multiple viruses including BBTV, BBrMV and SCMV, and with other viruses that may be infecting abaca, e.g. ABTV. In this study, BBTV, BBrMV and SCMV were detectable by ELISA and the method was

reliable enough for virus detection. ELISA proved to be a very reliable method for BBTV detection in micropropagated plants (Thomas et al. 1995). It can detect BBTV even in asymptomatic plantlets (Sta. Cruz et al. 2016).

Table 1. Viruses detected by enzyme-linked immunosorbent assay in *in-vitro* abaca tissue cultures.

Tissue Culture Sample [1]	Tissue Culture Line	Number of Virus Positive Samples/ Samples Tested		
		BBTV	**BBrMV**	**SCMV**
Batch 1	1514	nt [2]	10/10	9/10
	H61122	nt	3/3	3/3
	1541	nt	6/6	4/6
	D21511	nt	6/6	5/6
	L51417	nt	2/2	2/2
	N21478	nt	1/1	1/1
	V1128	nt	1/3	3/3
	E21516	nt	1/1	0/1
Batch 2	ST10	3/3	3/3	3/3
	ST11	3/3	8/8	5/8
	ST12	1/1	3/5	4/5
	ST13	0/3	4/4	3/4
	ST14	1/2	4/5	4/5
	ST17	3/3	1/3	2/3
	ST18	1/2	5/7	1/7
	ST19	0/1	3/3	2/3
	ST20	2/2	4/4	2/4
	ST22	1/3	2/6	6/6

[1] Batch 1 samples from PhilFIDA while Batch 2 from NARC; Banana bunchy top virus (BBTV); Banana bract mosaic virus (BBrMV); Sugarcane mosaic virus (SCMV);
[2] nt: not tested due to limited samples for testing

Differential infection of abaca tissue culture plantles following subsequent *in vitro* and *ex vitro* cultures

Differential infections with BBTV, BBrMV or SCMV of abaca plantlets during subsequent cultures were also observed. Analysis of differential infection was done using ST lines or batch 2 samples.

BBTV infection. Detection of BBTV in plantlets obtained from subsequent cultures was not always consistent (Table 2). The presence BBTV was consistently detected in two plantlets, ST10(4) and ST10(8) of line ST10 at the first *in vitro* subculture stage. Although the virus was not tested at the first *in vitro* culture, plantlet ST10 (10-1) which was derived from the same ST10 line was consistently BBTV positive at the *ex vitro* culture stage. The virus was also consistently detected in plantlets ST11(2) and ST11(5) at the first subculture, and plantlet ST11(2-1) derived from ST11(2) was consistently positive at the *ex vitro* culture. BBTV was also consistently detected in plantlets ST17(7), ST17(3), ST17(2). On the other hand, BBTV was not consistently detected in plantlets of lines ST18, ST20 and ST22 (Table 2). For instance, plantlet ST18(10) was BBTV positive at the first subculture. However, the virus was not detectable in ST18(9-1) when tested at the *ex vitro* culture. The plantlet ST20(5) and derived plantlet ST20(5-1) were differentially infected at the first *in vitro* subculture and *ex vitro* culture stages, respectively. Furthermore, plantlet ST12(1) and the derived plantlet ST12(1-1) were also differentially infected at the first subculture and *ex vitro* stages. Plantlets of lines ST13 and ST19 were consistently negative at the first *in vitro* and *ex vitro* culture stages.

Table 2. Banana bunchy top virus (BBTV) detected by ELISA in abaca plantlets following *in-vitro* and subsequent *ex-vitro* culture.

Tissue Culture Line	First *in-vitro* Subculture		*Ex-vitro* Culture	
	Plantlet	Reaction in ELISA	Plantlet	Reaction in ELISA
ST10	ST10(4)	+	4-1	nt
ST10	ST10(8)	+	8-1	nt
ST10	ST10(10)	nt	10-1	+
ST11	ST11(2)	+	2-1	+
ST11	ST11(5)	+	5-1	nt
ST17	ST17(7)	+	7-1	nt
ST17	ST17(3)	+	3-1	nt
ST17	ST17(2)	nt	2-1	+
ST18	ST18(10)	+	10-1	nt
ST18	ST18(9)	nt	9-1	-
ST20	ST20(5)	-	5-1	+
ST20	ST20(10)	+	10-1	nt
ST22	ST22(3)	-	3-1	nt
ST22	ST22(5)	+	5-1	nt
ST22	ST22(8)	nt	8-1	-
ST12	ST12(1)	-	1-1	+
ST13	ST13(4)	-	4-1	nt
ST13	ST13(1)	-	1-1	nt
ST13	ST13(6)	nt	6-1	-
ST19	ST19(3)	-	3-1	-

(+) positive to BBTV in enzyme-linked immunosorbent assay; nt-not tested due to limited samples for testing.

BBrMV infection. Differential infection of BBrMV was also observed. The BBrMV was consistently detected at the first and second subcultures in four plantlets, ST10(8), ST13(1), ST9(3) and ST20(5). Each of these plantlets was BBrMV positive when tested at the first *in vitro* culture and the plantlets, ST10(8-1), ST13(1-1), ST19(3-1) and ST20(5-1) derived at the from the second subculture were consistently virus positive. However, differential infection of BBrMV was observed in some plantlets during the first subculture and the subsequent culture. Plantlets which were infected at the first subculture generated plantlets which were also BBrMV infected as well as plants negative to the virus. For instance ST14(2) was BBrMV positive at the first subculture but the plantlets derived from second subculture had differential infection with one, ST14(2-1) of three plantlets was infected while the other two, ST14(2-2) and ST14(2-3) were not infected. Likewise, some plantlets ST12(5), ST17(7), ST18(5) had differential infection following subsequent cultures. These plantlets were negative to BBrMV infection at the first subculture but generated plantlets, ST12(5-1), ST17(7-1) and ST18(5-1) which were BBrMV infected at the second subculture stage.

On the other hand, the plantlet ST11(2) that was negative to BBrMV at the first subculture generated plantlets, ST11(2-1) and ST11(2-3) that were also virus negative as well as virus positive plantlet ST11(2-2) at the second subculture. Likewise, plantlet ST22(10) was negative at the first subculture but produced virus positive plantlet, ST22(10-1) at the second subculture. Table 3 not indicated

Table 3. Banana bract mosaic virus (BBrMV) detected by ELISA in abaca plantlets following two *in-vitro* subcultures.

Tissue Culture Line	First *in-vitro* Subculture		Second *in-vitro* Subculture	
	Plantlet	Reaction in ELISA	Plantlet	Reaction in ELISA
ST10	ST10(8)	+	8-1	+
ST13	ST13(1)	+	1-1	+
ST19	ST19(3)	+	3-1	+
ST20	ST20(5)	+	5-1	+
ST14	ST14(1)	+	1-1	+
ST14	ST14(2)	+	2-1	+
ST14	ST14(2)	+	2-2	-
ST14	ST14(2)	+	2-3	-
ST12	ST12(5)	-	5-1	+
ST17	ST17(7)	-	7-1	+
ST18	ST18(5)	-	5-1	+
ST11	ST11(2)	-	2-1	-
ST11	ST11(2)	-	2-2	+
ST11	ST11(2)	-	2-3	-
ST22	ST22(10)	-	10-1	+
ST22	ST22(10)	-	10-2	-
ST22	ST22(10)	-	10-3	-

(+) positive to BBrMV in enzyme-linked immunosorbent assay

SCMV infection. Detection of SCMV was not consistent following subsequent culture (Table 4). Although consistent SCMV infection was observed for some plantlets the other plantlets had differential infection. The presence of SCMV in plantlets of four lines ST10, ST12, ST13 and ST17 was consistent when tested at the first and second subcultures. For instance, plantlet ST10(8) at the first subculture stage and the plantlet ST10(8-1) derived from it were both virus positive. Likewise, plantlets ST12(5-1), ST13(1-1) and ST17(7-1) were virus positive. However, SCMV positive plantlet ST14(2) at the first subculture stage generated plantlets at the second subculture which had inconsistent reaction wherein ST14(2-1) was virus positive while ST14(2-2) and ST14(2-3) were negative. Plantlet ST19(3) was positive at the first subculture but produced plantlet ST19(3-1) that was virus negative at the second subculture. On the other hand, ST18(5) and ST20(5) were negative to the virus at the first subculture stage but produced plantlets, ST18(5-1) and ST20(5-1) which were virus positive at the second subculture. Although ST22(10) was virus negative at the first subculture, one of the plantlets ST22(10-1) produced at the second subculture was virus positive and the two plantlets ST22(10-2), ST22(10-3) were negative. Plantlet ST11(2) was consistently negative at the first and second subcultures.

The differential infection observed in this study can be attributed to the irregular distribution and movement of the virus in actively growing meristematic tissues as shown for BBTV (Thomas et al. 1995). Inconsistent transmission of BBTV in micropropagated banana was reported by Thomas and co-workers (1995). In this way, subculturing can result in the appearance of BBTV-free plantlets which were derived from virus infected plant. They found that nine-month extended subculturing can result in the appearance of BBTV-free plantlets which were derived from virus infected plant. This inconsistent transmission also means that the plants are differentially infected during *in vitro* culture. The differential infection of plantlets during tissue culture and the subsequent subcultures would

affect the reliability of virus indexing. The critical time of virus indexing then would be testing the tissue explants before they are subjected to the tissue culture process, and the tissue must be ensured to be virus-free using sensitive method like ELISA or PCR. Monitoring of virus infection during the tissue culture process is also necessary to ensure that the plantlets would be virus-free, particularly before they are used for the subsequent culture. Finally, virus indexing is needed at the *ex vitro* stage before the plantlets are finally taken out for field planting. Random sampling of about 1-10% of the plantlets which is usually practiced for virus indexing may not ensure that the plantlets that will be released would be virus-free. Thus, testing of more number of plantlets would be necessary, however an efficient system for mass indexing needs to be developed.

Table 4. Sugarcane mosaic virus (SCMV) detected by ELISA in abaca plantlets following two *in-vitro* subcultures.

Tissue Culture Line	First *in-vitro* Subculture		Second *in-vitro* Subculture	
	Plantlet	Reaction in ELISA	Plantlet	Reaction in ELISA
ST10	ST10(8)	+	8-1	+
ST12	ST12(5)	+	5-1	+
ST13	ST13(1)	+	1-1	+
ST17	ST17(7)	+	7-1	+
ST14	ST14(1)	+	1-1	+
ST14	ST14(2)	+	2-1	+
ST14	ST14(2)	+	2-2	-
ST14	ST14(2)	+	2-3	-
ST19	ST19(3)	+	3-1	-
ST18	ST18(5)	-	5-1	+
ST20	ST20(5)	-	5-2	+
ST22	ST22(10)	-	10-1	+
ST22	ST22(10)	-	10-2	-
ST22	ST22(10)	-	10-3	-
ST11	ST11(2)	-	2-1	-
ST11	ST11(2)	-	2-2	-
ST11	ST11(2)	-	2-3	-

(+) positive to SCMV in enzyme-linked immunosorbent assay

BBTV infection as detected by polymerase chain reaction (PCR) in *in vitro* abaca cultures

The presence of BBTV in *in vitro* and *ex vitro* cultures was confirmed by PCR using the BBT1/BBT2 primers which amplified a region of the DNA R component of the BBTV genome (Table 5). The expected PCR amplification product of 349 bp was obtained in the positive samples. BBTV was detected in all the lines tested. The virus was consistently detected in all plantlets of lines ST12(1,4,5), ST13(2,3,5) and ST18(2,4,5) of the undiluted DNA template. BBTV was also consistently detected in ST17, ST10 and ST11. However, the virus was detected when the template DNA was diluted at 1:10, except in ST17(4). On the other hand, the virus was not consistently detected in ST19 and ST14. For instance, BBTV was detected in ST19(1) and ST19(5) while ST19(2) was negative even in the undiluted template DNA.

BBTV infection of *in vitro* and *ex vitro* cultures was confirmed by PCR detection. The sensitivity of BBTV detection by PCR was affected by the dilution of template DNA. Detection of BBTV by PCR using DNA extracted from leaves of mature plant was found to be more efficient using

diluted (1:10 or 1:20 dilution) than undiluted samples (Sta. Cruz et al. 2016). This is because dilution of template DNA may have reduced the concentration some inhibitors allowing more efficient PCR detection. In this study, although the efficiency of PCR detection increased with template DNA dilution (1:10), most of the *in vitro* cultures were PCR positive even with undiluted DNA. However, this results need to be confirmed using more number of samples in the test.

Table 5. Banana bunchy top virus infection detected by PCR in *in-vitro* abaca cultures.

Tissue Culture Plantlet	Undiluted DNA template	1:10 template DNA dilution
ST12 (1)	+	nt
ST12 (4)	+	nt
ST12 (5)	+	nt
ST13 (2)	+	nt
ST13 (3)	+	nt
ST13 (5)	+	nt
ST18 (2)	+	nt
ST18 (4)	+	nt
ST18 (5)	+	nt
ST17 (4)	+	nt
ST17 (1)	-	+
ST17 (5)	-	+
ST10 (1)	-	+
ST10 (2)	-	+
ST11 (2)	-	+
ST19 (1)	+	nt
ST19 (5)	+	nt
ST19 (2)	-	-
ST14 (4)	+	nt
ST14 (2)	-	+
ST14 (5)	-	-

(+) positive to BBTV in polymerase chain reaction
nt-not tested due to limited samples for testing.

This study generated information useful for the establishment of reliable virus indexing of abaca tissue cultures. Since, multiple viruses are transmitted in *in vitro* cultures, it is suggested that the source of the explants must be tested for multiple infections to ensure that the explants are clean to prevent the multiplication of the virus during propagation through tissue culture. The explants can be tested by ELISA and confirmed by PCR for the presence of the virus. Further study to compare the sensitivity of ELISA and PCR is needed to determine which method is more efficient for virus indexing of tissue cultured abaca.

CONCLUSION

In this study, differential infections of BBTV causing bunchy top, as well as BBrMV and SCMV causing mosaic diseases were detected by enzyme-linked immunosorbent assay. The virus was

detected in plantlets obtained from a single tissue culture line, and in plantlets obtained following subsequent *in vitro* (first and second subculture) and *ex vitro* cultures. Infection occurred as mixtures of BBTV, BrMV and SCMV. Although the plantlets of each line were derived from a single explant, these plantlets were found to be differentially infected. Likewise, the plantlets obtained from a single plantlet following subsequent culture were also differentially infected. The differential infection resulted to inconsistency of virus detection by ELISA. Differential infection of BBTV was also detected by PCR. The sensitivity of BBTV detection by PCR increased with the dilution of template DNA. The information generated from this study will be useful for the establishment of a reliable method for virus indexing considering the differential virus infection of tissue culture plantlets.

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REFERENCES

Aspuria, E.T. 2003. Abaca tissue culture: its prospects for crop improvement through biotechnology (Philippines Univ. Los Banos, College, Laguna (Philippines). Dept. of Horticulture). http://agris.fao.org/agris-search/search.do?recordID=PH2004000267

Bajet, N.B. and L.V. Magnaye. 2002. Virus diseases of banana and abaca in the Philippines. Los Baños, Laguna: PARRFI. 82 p.

Diekmann, M. and C.A.J Putter. 1996. FAO/IPGRI Technical guidelines for the safe movement of germplasm; No. 15. Musa. 2nd edition. Rome Food and Agriculture Organization of the United Nations, Rome/International Plant Genetic Resources Institute.

Drew, R.A., J.A. Moisander and M.K. Smith. 1989. The transmission of banana bunchy top virus in micropropagated bananas. Plant Cell, Tissue and Organ Culture. 16:187-193.

Drew, R.A., M.K. Smith and D.W. Anderson. 1992. Field evaluation of micropropagated bananas derived from plants containing banana bunchy top virus. Plant Cell, Tissue and Organ Culture 28:203-2015.

Eloja, A.L., L.G. Velasco and J.A. Agati. 1962. Studies on the abaca mosaic disease I. Sap transmission of the abaca mosaic virus. Philip. Agric. 27:75-84.

Eloja, A.L. and T.W. Tinsley. 1963. Abaca mosaic virus and its relationship to sugarcane mosaic. Ann. Appl. Biol. 51:253-258.

Espino, T.M., S.B. Exconde, F.B. Zipagan and R.R.C. Espino. 1990. Banana bract mosaic, a new disease of banana. Philipp. Agric. 73:61-68.

[FIDA]. Fiber Industry Development Authority. 2011. Statistical bulletin for fiber industry. Retrieved April 28, 2013. www.fida.gov.ph.

[FIDA]. Fiber Industry Development Authority. 2012. FIDA Accomplishment Report. Retrieved December 15, 2013. www.fida.gov.ph

Furuya, N., T.O. Dizon and K.T. Natsuaki. 2006. Molecular characterization of *Banana bunchy top virus* and *Cucumber mosaic virus* from abaca (*Musa textilis* Nee). J Agric Sci, Tokyo University of Agriculture. 51(2):92-101.

Gambley, C.F., J.E. Thomas, L.V. Magnaye and L. Herradura. 2004. Abaca mosaic virus: a distinct strain of *Sugarcane mosaic virus.* Aust. Plant. Pathol. 33:475-484.

Hwang, S.C. and H.J. Su. 1998. Production and cultivation of virus-free banana tissue cultured Plantlets in Taiwan. Managing banana and citrus diseases. Proceedings of a regional workshop on disease management of banana and citrus through the use of disease-free planting materials. Davao, Philippines. 14–16 October 1998.

Magee, C.J. 1953. Some aspects of the bunchy top disease of banana and other Musa spp. J Proc. Royal Soc. New South Wales. 87:3–18.

Magnaye, L.V. and R.R.C. Espino. 1990. Banana bract mosaic, a new disease of banana. I. Symptomatology. Philipp. Agric. 73: 55-59.

Mahadev, S.R., S.K. Thamilarasan and A. Kathithachalam. 2013. PCR detection of banana bunchy top virus (BBTV) at tissue culture level for the production of virus-free planting materials. Int. Res. J. Biological Sci. 2(6):22-26.

Muñez, A.R. 1992. Symptomatology, transmission and purification of banana bract mosaic virus (BBrMV) in giant Cavendish banana. [MS thesis]. College, Laguna, Philippines: University of the Philippines Los Baños. 57 p. (Available at the UPLB Library).

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum. 15 (3): 473–497.

Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Science. 163:85 - 87.

Ocfemia, G.O. 1930. Bunchy-top of abaca or Manila hemp. I. A study of the cause of the disease and its method of transmission. Am. J. Bot. 17:1-18.

[PHILFIDA] Philippine Fiber Industry Development Authority. 2016. Fiber Statistics. http://www.philfida.da.gov.ph/index.php/2016-11-10-03-32-59/2016-11-11-07-56-39/fiber-statistics-2016

Pinili, M.S., T.O. Dizon and K.T. Natsuaki. 2011. Low divergence of banana bract mosaic virus infecting abaca and banana in the Philippines. The 4th Asian Conference on Plant Pathology concurrent with the 18th Biennial Australasian Plant Pathology Society Conference. April 26- 29, 2011. Darwin Convention Center. Darwin NT Australia.

Ramos, C.S. and A.B. Zamora. 1990. Elimination of banana bunchy top infection from banana (*Musa* sp. Cv. Lakatan) by heat treatment and meristem culture. Phil. J. Crop Sci. 16:119-123.

Raymundo, A.D. 2000. Saving abaca from the onslaught of the bunchy-top disease. Philipp. Agric. Sci. 83:379-385.

Rodoni, B.C., Y.S. Ahlawat, A. Varma, J.L. Dale and R.M. Harding. 1997. Identification and characterization of banana bract mosaic virus in India. Plant Disease. 81:66-672.

Sharman, M., C.F. Gambley, E.O. Oloteo, R.V.J. Abgona and J.E. Thomas. 2000. First record of natural infection of abaca (*Musa textilis*) with banana bract mosaic potyvirus in the Philippines. Australasian Plant Pathol. 29, 69. doi: 10.1071.AP00012.

Sharman, M., J.E. Thomas and T.A. Holton. 2008. *Abaca bunchy top virus*, a new member of the genus Babuvirus (family) Nanoviridae. Arch. Virol. 153:135-147.

Sta. Cruz, F.C., G.B. Belen and A.N. Alviar. 2016. Serological and molecular detection of mixed bunchy top and mosaic virus infections in abaca (*Musa textilis* Nee). Phil. Agric. Sci. 99(1):68-78.

Su H-J. 1999. Development and application of molecular diagnostic probes for detection, characterization, and management of banana viruses. in: advancing banana and plantain R and D in Asia and the Pacific. Proceedings of the 9th INIBAP-ASPNET Regional Advisory Committee meeting; 1999 November 2-5; Guangzhou, China: South China Agricultural University. p. 35-51.

Thomas, J.E. and R.G. Dietzgen. 1991. Purification, characterization and serological detection of virus-like particles associated with banana bunchy top disease in Aust. J. Gen. Virol. 72:217-224.

Thomas, J.E., M.K. Smith, A.F. Kessling and S.D. Hamil. 1995. Inconsistent transmission of banana bunchy top virus in micropropagated bananas and its implication for germplasm screening. Aust. J. Agric. 46:663-71.

Thomas J.E., A.D.W. Geering, G. Dahal, B.E.L. Lockhart and G. Thottappilly. 2003. Virus and virus-like diseases of major crops in developing countries in banana and plaintain. G. Loebenstein and G. Thottappilly (eds.). Kluwer Academic Publishers. pp 477–496.

Thompson, D. and R.G. Dietzgen. 1995. Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization. J. Virol. Methods 54:85-95.

Wu, R.Y. and H.J. Su. 1991. Regeneration of healthy banana plantlets from banana bunchy top virus infected tissues cultured at high temperature. Plant Pathology. 40:4-7.